



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|--|
| (51) International Patent Classification: C12N 9/64 | A1 | (11) International Publication Number: WO 96/26268 (43) International Publication Date: 29 August 1996 (29.08.96) |
| (21) International Application Number: PCT/US96/02420 (22) International Filing Date: 21 February 1996 (21.02.96) (30) Priority Data: 08/391,743 21 February 1995 (21.02.95) US (71) Applicant: GENZYME TRANSGENICS CORPORATION [US/US]; One Mountain Road, Framingham, MA 01701 (US). (72) Inventors: DiTULLIO, Paul; Apartment 68, 915 Edgell Road, Framingham, MA 01701 (US). MEADE, Harry; 62 Grasmere Street, Newton, MA 02154 (US). COLE, Edward, S.; 11 Cemetery Street, Mendon, MA 01751 (US). (74) Agents: GOSZ, William, G. et al.; Genzyme Corporation, One Mountain Road, Framingham, MA 01701 (US). | | (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> |
| (54) Title: TRANSGENICALLY PRODUCED ANTITHROMBIN III | | |
| (57) Abstract <p>This invention relates to transgenically produced human Antithrombin III (tgATIII). The human ATIII produced by the transgenic process of the present invention has a monosaccharide composition which comprises N-acetylgalactosamine (GalNAc) along with fucose, N-acetylglucosamine, galactose, mannose, and N-acetylneuraminic acid/N-glycolyneuraminic acid. The monosaccharide composition differs with that of plasma derived ATIII (phATIII). It has been found that tgATIII has an increased clearance rate when compared to phATIII.</p> | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|--|----|--------------------------|
| AM | Armenia | GB | United Kingdom | MW | Malawi |
| AT | Austria | GE | Georgia | MX | Mexico |
| AU | Australia | GN | Guinea | NE | Niger |
| BB | Barbados | GR | Greece | NL | Netherlands |
| BE | Belgium | HU | Hungary | NO | Norway |
| BF | Burkina Faso | IE | Ireland | NZ | New Zealand |
| BG | Bulgaria | IT | Italy | PL | Poland |
| BJ | Benin | JP | Japan | PT | Portugal |
| BR | Brazil | KE | Kenya | RO | Romania |
| BY | Belarus | KG | Kyrgyzstan | RU | Russian Federation |
| CA | Canada | KP | Democratic People's Republic of Korea | SD | Sudan |
| CF | Central African Republic | KR | Republic of Korea | SE | Sweden |
| CG | Congo | KZ | Kazakhstan | SG | Singapore |
| CH | Switzerland | LI | Liechtenstein | SI | Slovenia |
| CI | Côte d'Ivoire | LK | Sri Lanka | SK | Slovakia |
| CM | Cameroon | LR | Liberia | SN | Senegal |
| CN | China | LT | Lithuania | SZ | Swaziland |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | LV | Latvia | TG | Togo |
| DE | Germany | MC | Monaco | TJ | Tajikistan |
| DK | Denmark | MD | Republic of Moldova | TT | Trinidad and Tobago |
| EE | Estonia | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | UG | Uganda |
| FI | Finland | MN | Mongolia | US | United States of America |
| FR | France | MR | Mauritania | UZ | Uzbekistan |
| GA | Gabon | | | VN | Viet Nam |

TRANSGENICALLY PRODUCED ANTITHROMBIN III

Background of the Invention

5 A growing number of recombinant proteins are being developed for therapeutic and diagnostic applications; however, many of these proteins may be difficult or expensive to produce in a functional form in the required quantities using conventional methods. Conventional methods involve inserting the gene
10 responsible for the production of a particular protein into host cells such as bacteria, yeast, or mammalian cells, and then growing the cells in culture media. The cultured cells then synthesize the desired protein. Traditional bacteria or yeast systems may be unable to produce many complex proteins in a
15 functional form. While mammalian cells can reproduce complex proteins, they are generally difficult and expensive to grow, and produce only mg/L quantities of protein.

 The application of transgenic technology to the commercial production of recombinant proteins in the milk of transgenic
20 animals offers significant advantages over traditional methods of protein production. These advantages include a reduction in the total amount of required capital expenditures, elimination of the need for capital commitment to build facilities early in the product development life cycle, and lower direct production cost
25 per unit for complex proteins. Of key importance is the likelihood that, for certain complex proteins, transgenic production may represent the only technologically and economically feasible method of commercial production.

 Antithrombin III (ATIII) is a serine protease inhibitor which
30 inhibits thrombin and the activated forms of factors X, VII, IX, XI, and XII. It is normally present in serum at levels of 14-20 mg/dL. Decreased levels of ATIII may be found in the serum of individuals who have either a hereditary deficiency of ATIII or an acquired deficiency, which can result from a number
35 of pathologic conditions. The conventional treatment for

hereditary ATIII deficiency is protein replacement therapy, which may also be effective in treating some acquired deficiencies.

Current methods of obtaining ATIII involves isolating the protease inhibitor from blood plasma. However, the use of plasma-based ATIII presents various problems due to the many components in plasma, including: variation between lots; immunogenicity problems; and biohazardous risks due to viral contamination.

A need exists to develop a method to produce ATIII without the inherent problems of the present process.

Summary of the Invention

This invention relates to transgenically produced human Antithrombin III (tgATIII). The human ATIII (hATIII) produced by the transgenic process of the present invention has a monosaccharide composition which comprises N-acetylgalactosamine (GalNAc) along with fucose, N-acetylglucosamine, galactose, mannose, and N-acetylneuraminic acid/N-glycolyneuraminic acid. The monosaccharide composition differs with that of human plasma derived ATIII (phATIII). It has been found that tgATIII has an increased clearance rate when compared to phATIII.

Brief Description of the Drawings

Figure 1 is a chromatograph comparing the HPLC profile of the tgATIII of the present invention and plasma derived ATIII.

Figure 2 is a photograph of a SDS page gel showing a greater than 98% purity of tgATIII.

Figure 3 illustrates that the disulfide cross-linking between phATIII and the tgATIII. The peaks labelled with the arrows are the peaks containing disulfide cross-linked peptides.

Figure 4 shows the individual equivalent peptides of tgATIII and phATIII by mass spectrometry.

Figure 5 illustrates the mass spectrometry data for one of the individual glycopeptide residues from plasma derived ATIII.

5 Figure 6 shows the mass spectrometry data for the same glycopeptide residue as shown for Figure 5, but for tgATIII.

Figure 7 illustrates the sialic acid composition of human ATIII, goat ATIII, tgATIII, and transgenic mouse ATIII (tmATIII).

10 Figure 8 shows a graph comparing the clearance rate of phATIII and tgATIII in mice.

Figure 9 shows a graph comparing the clearance rate of phATIII and tgATIII in monkey.

Detailed Description of the Invention

15

This invention is based upon the discovery that hATIII produced by the transgenic method of the present invention is structurally different than ATIII extracted from plasma (phATIII). The monosaccharide composition of the transgenically produced
20 ATIII (tgATIII) differs in the composition and the quantity when compared to plasma derived ATIII. In particular, the glycosylation sites on the tgATIII vary considerably in contrast to phATIII which are all uniform.

Exemplification

Generation of the gene construct

A mammary gland-specific transgene was constructed by inserting the human Antithrombin III (hATIII) cDNA into the
30 caprine beta casein gene (CSN2). The caprine beta casein gene was cloned as an 18.5 Kb fragment in a lambda EMBL3 vector (Roberts, *et al.*, Gene., 1992. 121: p. 255-262). The 6.2 Kb promoter (including exon 1 and part of exon 2) was fused to the hATIII cDNA to direct high level mammary gland-specific
35 expression. A 7.2 Kb 3' flanking region (including part of exon 7, exon 8, and exon 9) was added to the 3' end of the hATIII cDNA to

help stabilize the expression levels. The 14.95 Kb transgene was excised from bacterial sequences and injected into goat embryos for the production of hATIII in goats' milk.

5 **Identification of gene coding for the protein of interest**

The hATIII cDNA was received from Dr G. Zettlmeißl (Behringwerke A.G., Marburg, Germany) in the plasmid pBAT6. The sequence of the cDNA is the same as that published by Bock, *et al.*, Nucleic Acids Research, 1982. 10: p. 8113-8125, except for
10 the silent nucleotide changes at bp 1096 (T - C) and bp 1417 (A - G).

Identification of regulatory sequences of interest

To direct high level tissue-specific expression of hATIII to
15 the mammary gland of transgenic goats, the goat beta casein gene was cloned from a lambda EMBL3 goat genomic library. The goat beta casein gene is a mammary gland-specific gene which directs expression of high levels of beta casein into the milk. In goats, beta casein is thought to comprise 25-50% of the total milk
20 proteins (~10-20 mg/ml). The goat beta casein gene was cloned from a Saanen goat genomic library and characterized in transgenic mice as described in Roberts, *et al.*, 1992.

Cloning DNA fragments

25 High molecular weight goat DNA was isolated from a Saanen goat blood sample according to the procedure described by Herrmann and Frischauf, Methods Enzymology, 1987. 152: p. 180-183. The genomic DNA was partially digested with MboI and ligated into BamHI cleaved lambda EMBL3 phage arms (Stratagene,
30 La Jolla, CA) by standard methods (Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press, 1989). The Saanen goat genomic library consisted of 1.2×10^6 recombinant phage and was used to isolate the goat beta casein gene. The goat genomic library was screened using a 1.5

Kb HindIII/TthIII1 fragment encoding the entire mouse CSN2 cDNA (Yoshimura, *et al.*, Nucleic Acids Res., 1986. 14: p. 8224) and three overlapping clones were identified. The three overlapping clones were designated EMBL 3-7, 3-8, and 3-11. Only clone 3-11
5 contained the entire goat beta casein gene and was used for all future analysis and vector construction (Roberts, *et al.*, 1992).

Characterization of cloned material

The goat beta casein gene was isolated as an 18.5 Kb
10 fragment in lambda EMBL3 phage clone designated EMBL3-11 (Roberts, *et al.*, 1992). The fragment contains 4.2 Kb of 5' flanking region, exons 1 through 9, and 5.3 Kb of 3' flanking sequence. The gene was subcloned into pUC19 or pGEM3Z vectors. The subclones were sequenced by Sanger dideoxy sequencing to
15 verify all intron/exon boundaries and the sequence is shown in SEQ. ID. No.: 1. All sequences are deposited with GenBank (accession Nos. M90556, M90557, M90558, M90559, M90560, M90561, and M90562).

20 Assembly and characterization of the gene construct.

The beta casein hATIII transgene was constructed to resemble as accurately as possible the intact goat beta casein gene. The vector was 14.95 Kb in length with 4.2 Kb of 5' flanking
25 sequence and 5.3 Kb of 3' flanking sequence. The vector was engineered with three introns between exons 1-2, 7-8, and 8-9 to allow for splicing of the transcript. The introns were added to increase expression of the transgene (Brinster *et al.*, Proc. Natl. Acad. Sci. USA., Devel. Biol. 85, pp.836-840, 1988.) and the 3'
30 flanking sequence to decrease chromosomal position effects.

To allow for the cloning of various inserts, the beta casein promoter and 3' flanking region were reassembled from the subclones with unique restriction sites in exon 2 and exon 7. To remove the initiator methionine of the goat beta casein, the
35 plasmid Bc106 was digested with the restriction enzyme TaqI.

The TaqI site restricts the plasmid Bc106 six nucleotides upstream of the initiator methionine (TaqI site marked with ****, Figure 10). Following digestion with TaqI, the ends were filled in with Klenow DNA polymerase and a BamHI linker ligated into place. The ligation mixture was extracted with phenol-chloroform, chloroform-isoamyl alcohol, and ethanol precipitated as described in Maniatis, *et al.*, 1989. The DNA was resuspended in digestion buffer and digested with EcoRI and BamHI. The resulting 1.5 Kb fragment was gel purified and ligated into pUC to form the plasmid Bc150. The entire available 5' region of the beta casein gene was constructed by sequential addition of fragments contained in the subclones Bc104, Bc147, and Bc103. The orientation of the Bc147 clone was verified by restriction mapping. The replacement of the 2.6Kb XbaI fragment with the XbaI fragment from Bc147 was needed to replace a 31bp deletion in the promoter between adjacent EcoRI restriction sites. To allow for the addition of the human antithrombin III cDNA, the BamHI site on the 3' end of Bc113 was converted to an XhoI site to form Bc114.

20 The 3' end of the goat beta casein gene was constructed in a similar fashion to the 5' end. The 1.8 Kb Bc107 clone was restricted with PpuMI to allow for the addition of a BamHI linker. The BamHI site was placed in exon 7 to include the intron between exons 7 and 8. The BamHI site is seven amino acids 5' to the termination of the goat beta casein mRNA but will have no effect on the hATIII since the cDNA for ATIII contains a termination signal. The BamHI/HindIII fragment spanning exon 7 from Bc107 was ligated into the vector Bc109 which contained the extreme 3' HindIII/SalI fragment to form Bc165. The 3' end was completed by the addition of the 4.4 Kb HindIII fragment from Bc108 to form the vector Bc118. The orientation of HindIII fragment from Bc108 was verified by restriction mapping. To allow for the addition of the hATIII cDNA, the 5' BamHI site in the vector Bc118 was changed to XhoI and the 3' SalI was converted to NotI to form Bc122.

In order to clone the entire hATIII cDNA into the goat beta casein expression vector, the 5' EcoRI restriction site was converted to an XhoI site using site-directed mutagenesis. The new vector designated pAT7 was sequenced to confirm the addition of the Sall site and the absence of any nucleotide changes in the 5' end. The hATIII cDNA was isolated as a 1.45 Kb XhoI/Sall fragment for cloning into the beta casein expression vector.

The 1.45KB XhoI/Sall hATIII cDNA was first ligated into XhoI digested Bc114 and Bc122 to form Bc143 and Bc144, respectively. The orientation of the cDNA was confirmed by sequencing and restriction analysis. The unique SacII restriction in the hATIII cDNA allows for the directional cloning of the intact goat beta casein hATIII vector. The complete transgene was assembled by ligating the Sall/SacII fragment from Bc143 into a Sall/SacII digested Bc144. The 5' and 3' junction of the hATIII cDNA were confirmed by sequencing to ensure no mutations had occurred. The final vector (Bc6) was 14.95 Kb in length and contained 4.2 Kb of 5' flanking region, exon 1, intron 1, part of exon 2, the 1.45 Kb human AT III cDNA, part of exon 7, intron 7, exon 8, intron 8, exon 9, and 5.3 Kb of 3' flanking sequence. The transgene was excised from the pUC backbone by digestion with Sall/NotI. The Bc6 transgene directs mammary specific expression of an approximately 1.5Kb hATIII-goat beta casein hybrid mRNA containing the 5' and 3' untranslated regions of the goat beta casein mRNA and coding region of the hATIII mRNA.

Testing and characterization of gene constructs

Transgene constructs are generally tested in a mouse model system to assess their ability to direct high levels of expression and their ability to express in a tissue-specific manner.

The Bc6 transgene was prepared for microinjection by digestion of the Bc6 plasmid with Sall/NotI, removal of the bacterial sequences by TAE agarose gel electrophoresis followed by banding of the transgene on a cesium chloride gradient

according to Lacy, *et al.*, A Laboratory Manual, 1986. Cold Springs Harbor Laboratory, NY. The 14.95 Kb transgene was then microinjected into mouse embryos to test the ability of the goat beta casein promoter to direct high level expression of recombinant human Antithrombin III (rhATIII) into the milk. Seventeen transgenic mouse lines were generated as determined by Southern analysis. One mouse line showed a level of expression of rhAT III of 0.7-1.0 mg/ml as determined by Western blot analysis and a thrombin inhibition assay. Based on expression results and data collected from other ongoing mouse and goat experiments, the Bc6 transgene was microinjected into goat embryos for production of transgenic goats.

Generation and characterization of transgenic animals

A founder (F_0) transgenic goat is defined as a viable transgenic animal resulting from embryo transfer of fertilized goat eggs that have been microinjected with a specified construct (Bc6). Four founder Bc6 goats were produced. The general methodologies that follow in this section were used to generate all transgenic goats.

Goat Species and breeds:

The transgenic goats produced for ATIII production are of Swiss origin, and are the Alpine, Saanen, and Toggenburg breeds.

Goat superovulation:

The timing of estrus in the donors was synchronized on Day 0 by 6 mg subcutaneous norgestomet ear implants (Syncromate-B, CEVA Laboratories, Inc., Overland Park, KS). Prostaglandin was administered after the first seven to nine days to shut down the endogenous synthesis of progesterone. Starting on Day 13 after insertion of the implant, a total of 18 mg of follicle-stimulating hormone (FSH - Schering Corp., Kenilworth, NJ) was given

intramuscularly over three days in twice-daily injections. The implant was removed on Day 14. Twenty-four hours following implant removal the donor animals were mated several times to fertile males over a two-day period (Selgrath, et al.,
5 Theriogenology, 1990. pp. 1195-1205).

Embryo collection:

Surgery for embryo collection occurred on the second day
10 following breeding (or 72 hours following implant removal). Superovulated does were removed from food and water 36 hours prior to surgery. Does were administered 0.8 mg/kg Diazepam (Valium®), IV, followed immediately by 5.0 mg/kg Ketamine (Keteset), IV. Halothane (2.5%) was administered during surgery
15 in 2 L/min oxygen via an endotracheal tube. The reproductive tract was exteriorized through a midline laparotomy incision. Corpora lutea, unruptured follicles greater than 6 mm in diameter, and ovarian cysts were counted to evaluate superovulation results and to predict the number of embryos that
20 should be collected by oviductal flushing. A cannula was placed in the ostium of the oviduct and held in place with a single temporary ligature of 3.0 Prolene. A 20 gauge needle was placed in the uterus approximately 0.5 cm from the uterotubal junction. Ten to twenty ml of sterile phosphate buffered saline (PBS) was
25 flushed through the cannulated oviduct and collected in a Petri dish. This procedure was repeated on the opposite side and then the reproductive tract was replaced in the abdomen. Before closure, 10-20 ml of a sterile saline glycerol solution was poured into the abdominal cavity to prevent adhesions. The linea alba
30 was closed with simple interrupted sutures of 2.0 Polydioxanone or Supramid and the skin closed with sterile wound clips.

Fertilized goat eggs were collected from the PBS oviductal flushings on a stereomicroscope, and were then washed in Ham's F12 medium (Sigma, St. Louis, MO) containing 10% fetal bovine
35 serum (FBS) purchased from Sigma. In cases where the pronuclei were visible, the embryos were immediately microinjected. If

pronuclei were not visible, the embryos were placed in Ham's F12 containing 10% FBS for short term culture at 37°C in a humidified gas chamber containing 5% CO₂ in air until the pronuclei became visible (Selgrath, et al., Theriogenology, 1990. p. 1195-1205).

5

Microinjection procedure

One-cell goat embryos were placed in a microdrop of medium under oil on a glass depression slide. Fertilized eggs
10 having two visible pronuclei were immobilized on a flame-polished holding micropipet on a Zeiss upright microscope with a fixed stage using Normarski optics. A pronucleus was microinjected with the Bc6 DNA construct in injection buffer (Tris-EDTA) using a fine glass microneedle (Selgrath, et al.,
15 Theriogenology, 1990. p. 1195-1205).

Embryo development:

After microinjection, the surviving embryos were placed in
20 a culture of Ham's F12 containing 10% FBS and then incubated in a humidified gas chamber containing 5% CO₂ in air at 37°C until the recipient animals were prepared for embryo transfer (Selgrath, et al., Theriogenology, 1990. p. 1195-1205).

Preparation of recipients:

Estrus synchronization in recipient animals was induced by 6 mg norgestomet ear implants (Syncromate-B). On Day 13 after
insertion of the implant, the animals were given a single non-
30 superovulatory injection (400 I.U.) of pregnant mares serum gonadotropin (PMSG) obtained from Sigma. Recipient females were mated to vasectomized males to ensure estrus synchrony (Selgrath, et al., Theriogenology, 1990. p. 1195-1205).

35

Embryo Transfer:

5 All embryos from one donor female were kept together and transferred to a single recipient when possible. The surgical procedure was identical to that outlined for embryo collection outlined above, except that the oviduct was not cannulated, and the embryos were transferred in a minimal volume of Ham's F12 containing 10% FBS into the oviductal lumen via the fimbria using a glass micropipet. Animals having more than six to eight 10 ovulation points on the ovary were deemed unsuitable as recipients. Incision closure and post-operative care were the same as for donor animals (Selgrath, et al., Theriogenology, 1990. p. 1195-1205).

15 Monitoring of pregnancy and parturition:

Pregnancy was determined by ultrasonography 45 days after the first day of standing estrus. At Day 110 a second ultrasound exam was conducted to confirm pregnancy and assess fetal 20 stress. At Day 130 the pregnant recipient doe was vaccinated with tetanus toxoid and Clostridium C&D. Selenium and vitamin E (Bo-Se) were given IM and Ivermectin was given SC. The does were moved to a clean stall on Day 145 and allowed to acclimatize to this environment prior to inducing labor on about 25 Day 147. Parturition was induced at Day 147 with 40 mg of PGF_{2a} (Lutalyse®) purchased from Upjohn Company, Kalamazoo Michigan. This injection was given IM in two doses, one 20 mg dose followed by a 20 mg dose four hours later. The doe was under periodic observation during the day and evening following 30 the first injection of Lutalyse® on Day 147. Observations were increased to every 30 minutes beginning on the morning of the second day. Parturition occurred between 30 and 40 hours after the first injection. Following delivery the doe was milked to collect the colostrum and passage of the placenta was confirmed.

35

Verification of the transgenic nature of F₀ animals:

To screen for transgenic F₀ animals, genomic DNA was isolated from two different cell lines to avoid missing any mosaic transgenics. A mosaic animal is defined as any goat that does not have at least one copy of the transgene in every cell. Therefore, an ear tissue sample (mesoderm) and blood sample were taken from a two day old F₀ animal for the isolation of genomic DNA (Lacy, et al., A Laboratory Manual, 1986, Cold Springs Harbor, NY; and Herrmann and Frischaut, Methods Enzymology, 1987. 152: pp. 180-183). The DNA samples were analyzed by the polymerase chain reaction (Gould, et al., Proc. Natl. Acad. Sci, 1989. 86:p. 1934-1938) using primers specific for hATIII and by Southern blot analysis (Thomas, Proc Natl. Acad. Sci., 1980. 77:5201-5205) using a random primed hATIII cDNA probe (Feinberg and Vogelstein, Anal. Bioc., 1983. 132: pp. 6-13). Assay sensitivity was estimated to be the detection of one copy of the transgene in 10% of the somatic cells.

20 Generation and Selection of production herd

The procedures described above were utilized for production of the transgenic founder (F₀) goats, as well as other transgenic goats in our herd. The transgenic F₀ founder goats, for example, were bred to produce milk, if female, or to produce a transgenic female offspring if it was a male founder.

This transgenic founder male, was bred to non-transgenic females, and produced transgenic female offspring.

30 Transmission of transgene and pertinent characteristics

Transmission of the Bc6 transgene in our goat line was analyzed in ear tissue and blood by PCR and Southern blot analysis. For example, Southern blot analysis of the founder male and the three transgenic offspring showed no rearrangement or change in the copy number between generations. The Southern

blots were probed with the 1.45 Kb ATIII cDNA and a 0.38 Kb probe corresponding to goat beta casein exon 7. The blots were analyzed on a Betascope 603 and copy number determined by comparison of the transgene to the goat beta casein endogenous gene.

Evaluation of expression levels

The expression level of rhATIII in the milk of transgenic animals is determined using a thrombin inhibition assay, which measures the inhibition of thrombin's ability to remove a small peptide from an artificial substrate (S2238, Kabi, Franklin OH). The basis for this assay is described as follows. The interaction between ATIII and thrombin amounts to rapid irreversible inhibition of the protease by ATIII in the presence of heparin. However, the interaction is very slow in the absence of heparin. Attempts to extend the range of ATIII detectable on a single standard curve reveal that ATIII can only be determined accurately in stoichiometric titration across the linear range of standard curves. At low total thrombin concentration ($0.7 \times 10^{-9}\text{M}$), the effective measuring range for ATIII is $0.15 - 0.75 \times 10^{-9}\text{M}$ ($\sim 7.3 - 36.8 \text{ ng/ml}$). At high total thrombin, the effective measuring range for AT III is 0.25 to $1.25 \times 10^{-9}\text{M}$ ($\sim 12 - 60 \text{ ng/ml}$) if the data are fit with a first degree polynomial, and 0.25 to $2.5 \times 10^{-9}\text{M}$ ($\sim 12 - 120 \text{ ng/ml}$) if the data are fit with a second degree polynomial.

AT III activity assay

30 Materials:

1. 12 x 75 mm Glass Tubes;
2. Behring hATIII dilute to 0.5 mg/ml with diH_2O ($E_{0.1\%}^{280} = 0.72$);

3. Thrombin (100 units/vial diluted to 1.82 mls with 0.1 mg/ml BSA), purchased from Calbiochem, San Diego CA). Final Concentration 20 µg/ml;
4. Heparin (Sigma) from porcine intestinal mucosa 50 mg dissolved 5.0 mls diH₂O. Final Concentration 10 mg/ml;
5. Substrate (Kabi) S2238 25 mg diluted to 10 mls with diH₂O;
6. Dilution Buffer: 50 mM Tris, 150 mM NaCl, 0.1 mg/ml BSA pH 8.1;
7. Glacial Acetic Acid;
- 10 8. Repeat Pipettor; and
9. 37°C circulating H₂O bath.

ASSAY SET-UP

15 Dilution of Stocks:

1. AT III stock to 1 µg/ml: 20µl ATIII + 980 µls dilution buffer then 100µl + 900µl dilution buffer;
2. Heparin/Buffer: 12.5µl Heparin + 25 mls dilution buffer; and
- 20 3. Thrombin stock: 1:10 dilution into dilution buffer (2.0 mls for a 30 tube assay).

Standard Curve Set-Up

| 25 | Final Concentration AT III | Diluted AT III Stock | Dil Buffer | Heparin Buffer | Thrombin |
|----|----------------------------|----------------------|------------|----------------|----------|
| | 0 ng/ml | 0 µl | 100 µl | 750 µl | 50 µl |
| | 5 ng/ml | 5 µl | 95 µl | 750 µl | 50 µl |
| | 10 ng/ml | 10 µl | 90 µl | 750 µl | 50 µl |
| | 20 ng/ml | 20 µl | 80 µl | 750 µl | 50 µl |
| 30 | 30 ng/ml | 30 µl | 70 µl | 750 µl | 50 µl |
| | 40 ng/ml | 40 µl | 60 µl | 750 µl | 50 µl |
| | 50 ng/ml | 50 µl | 50 µl | 750 µl | 50 µl |
| | 60 ng/ml | 60 µl | 40 µl | 750 µl | 50 µl |

35

1. Incubate the above at 37°C for 15 minutes;
 2. Then add 100 µl of substrate to each tube using a repeat pipettor and incubate for 15 minutes at 37°C;
 - 5 3. Stop with 50 µl of glacial acetic acid to each tube; and
 4. Read at 405 nm. Blank with 150 µl Buffer, 750 µl Heparin Buffer, 100µl substrate and 50 µl Acetic acid stopped at time zero (NOT INCUBATED).
- 10 Calculations: Use a second order polynomial curve fit for the standard curve, plug A 405 nm readings into the equation. Divide by the volume of sample used in the assay tube in mls and multiply by the appropriate inverse dilution factor for the sample.

15

Characterization of Human ATIII Produced in the Milk of a Transgenic Goat.

- TgATIII was purified from goat milk by adaptation of methods previously developed for extracting ATIII from mouse milk and human blood. Other more novel methods for separating proteins from milk are also in development. The method described is the earliest method that we utilized. Milk from one BC6 goat contained tgATIII at a concentration estimated to be 0.08 mg/ml by ELISA. Other goats with much higher expression levels (4 to 6 mg/ml) were also developed and milk from those goats was purified using similar methods. Small scale purification was performed by the following method.

30

ATIII Purification Process

ATIII Extraction/Clarification

35

Thaw Milk
Add an equal volume of 2M Arginine
Centrifuge at 8000 rpm for 30 minutes and remove the fat pad on the top
Filter through a 0.2µm nominal polypropylene filter
Dilute the conductivity to less than 20 mS/cm (approximately a 1/7 dilution)
Expected step yield of AT III is ≥95%

40

Heparin-Hypar D™ Chromatography

- 5 Equilibrate with 3 column volumes of 20mM sodium phosphate, 50mM NaCl, pH7.0
 Load, the target operational capacity is approximately 8 mg of ATIII/mL of resin
 Wash with 4 column volumes of Equilibration Buffer
 Step Elute with 4 column volumes of 20mM sodium phosphate, 3.0M NaCl, pH7.0
 Dilute with 9 volumes of 1.5 M sodium citrate
 Expected step yield of AT III is ≥95%

10 Ether-Poros™ 50 Chromatography

- Equilibrate with 3 column volumes of 1.40M sodium citrate, pH7.0
 Load, the target operational capacity is approximately 4 mg of ATIII/mL of resin
 Wash with 4 column volumes of Equilibration Buffer
 15 Step Elute with 4 column volumes of 1.10M sodium citrate, pH7.0
 Expected step yield of AT III is ≥95%

Ultrafiltration with Amicon YM10 Spiral Crossflow

- 20 Concentrate to approximately 20 mg/mL (160 units/mL) and buffer exchange into 10 mM ammonium bicarbonate, pH 7.4.
 Expected step yield of AT III is ≥95%

Lyophilize

- 25 Reconstitute into buffer of choice
 Expected step yield of AT III is ≥95%

- 30 A sample of the concentrated pool was examined by SDS-PAGE (10% separating gel) with a human ATIII standard for comparison and estimation of purity. The purity is estimated to be >98% (see Figure 2.). The UV/Vis spectrum of tgATIII is indistinguishable (within experimental error) from that of plasma derived hATIII.
- 35 N-terminal sequence analysis gave the predicted sequence for hATIII. Reversed phase HPLC analysis showed three peaks for goat tgATIII compared to two peaks for plasma ATIII (Figure 1). All three peaks were found to be ATIII by N-terminal sequence analysis. Peak #1 is miniscule in the tgATIII and not apparent in
- 40 the plasma derived ATIII. Peak 2 is present in both samples and varies with preparations. Peak three is the predominant peak. Examination of specific peaks from Figure 1 following digestion with lysylendopeptidase and separation by reverse phase HPLC to create peptide maps identified the presence of oxidized
- 45 methionine residues in the two early small peaks of the tgATIII. The middle peak contained oxidized methionines mainly on a single peptide (K30 on table 2), whereas the earliest peak

contained oxidized methionines on most of the peptides that contained methionine. The major peak did not contain any oxidized methionines as measured by the technique being used above. The presence of oxidized methionines on this ATIII may be

5 a function of the processing of the molecule following removal of the milk from the goat.

Monosaccharide compositional analysis indicating the amounts of fucose (Fuc), GalNac, N-Acetylglucosamine (GlcNAc), Galactose (Gal), Mannose (Man) and Sialic Acid is shown below in

10 Table 1.

Table 1

| mole sugar/mole protein | | | | | | |
|-------------------------|------|--------|--------|-------|-------|-------------|
| Sample | Fuc | GalNAc | GlcNAc | Gal | Man | Sialic Acid |
| phATIII | 0.39 | 0.00 | 20.70 | 11.74 | 14.90 | 11.80 |
| tATIII | 3.18 | 2.39 | 14.75 | 6.44 | 18.18 | 5.21 |

15 Lysylendopeptidase maps of plasma derived hATIII and tgATIII have been analyzed by liquid chromatography/mass spectrometry (LC/MS) to compare the amino acid backbone structure, the glycosylation profile and to confirm the identity of the disulfide linked peptides. For non-reduced human plasma

20 ATIII and tgATIII, 32 of the 35 predicted peptides were identified, including the three pairs of disulfide linked peptides. The disulfide cross-linking was identical in the plasma ATIII and the tgATIII as shown in the peptide maps of the non-reduced proteins (Figure 3). The peaks labeled with the arrows are the

25 peaks containing disulfide cross-linked peptides. These peaks have identical retention times for each disulfide crosslinked peptide pair of each protein. The remaining peaks on the maps are identical with the exception of the glycosylated peptides. The maps of the reduced, pyridylethylated ATIII digests detail this

30 variability more specifically.

For reduced and pyridylethylated plasma and transgenic hATIII, 33 of the 35 predicted peptides were identified by LC/MS. Chromatograms of the plasma ATIII and the individual forms of the tgATIII can be seen in Figure 4 with the identification of the individual equivalent peptides by mass spectrometry shown in
5 Table 2 below. Again, all of the peptides are identical with the exception of the glycosylated peptides.

10

15

20

25

30

35

Table 2

LC/MS ANALYSIS of ATIII PEPTIDE MAPS

| PEPTIDE | RESIDUES | THEORETICAL MASS | phATIII | tgATIII |
|---------|----------|---------------------|---------|---------|
| K1 | 1-11 | 1232.4 | 1232.0 | 1232.3 |
| K2 | 12-28 | 2152.6 | 2152.6 | 2152.3 |
| K3 | 29-29 | 146.2 | ID | ID |
| K4 | 30-39 | 1093.1 | 1092.9 | 1092.8 |
| K5 | 40-53 | 1699.0 | 1698.7 | 1698.7 |
| K6 | 54-70 | 1957.1 | 1956.9 | 1956.9 |
| K7 | 71-91 | 2299.6 | 2299.3 | 2299.4 |
| K8 | 92-107 | GP | GP | GP |
| K9 | 108-114 | 838.9 | 838.7 | 838.7 |
| K20 | 115-125 | 1340.5 | 1340.3 | 1340.2 |
| K11 | 126-133 | 1170.5 | 1170.1 | 1170.1 |
| K12 | 134-136 | GP | GP | GP |
| K13 | 137-139 | 320.4 | ND | ND |
| K14 | 140-150 | 1219.4 | 1219.1 | 1219.4 |
| K15 | 151-169 | GP | GP | GP |
| K16 | 170-176 | 860.0 | 860.2 | 860.0 |
| K17 | 177-188 | 1330.4 | 1330.4 | 1330.2 |
| K18 | 189-193 | GP | GP | GP |
| K19 | 194-222 | 3248.8 | 3248.8 | 3248.8 |
| K20 | 223-226 | 502.6 | 502.9 | 502.8 |
| K21 | 227-228 | 233.3 | ND | ND |
| K22 | 229-236 | 978.1 | 978.1 | 977.9 |
| K23 | 237-241 | 698.8 | 698.5 | 698.6 |
| K24 | 242-257 | 1799.0 | 1798.8 | 1798.9 |
| K25 | 258-275 | 2209.6 | 2209.3 | 2209.6 |
| K26 | 276-287 | 1314.6 | ID | ID |
| K27 | 288-290 | 372.4 | ID | ID |
| K28 | 291-294 | 417.5 | 417.3 | 417.5 |
| K29 | 295-297 | 374.5 | 374.2 | 374.2 |
| K30 | 298-332 | 4261.0 | 4261.1 | 4260.9 |
| K31 | 333-348 | 1849.1 | 1849.0 | 1848.9 |
| K32 | 349-350 | 233.3 | ID | ID |
| K33 | 351-370 | 2202.5 | 2202.5 | 2202.2 |
| K34 | 371-403 | 3448.9 | 3448.7 | 3448.7 |
| K35 | 404-432 | 3421.2 | 3421.2 | 3421.2 |
| K3-4 | 29-39 | 1221.3 | 1221.4 | 1221.1 |
| K26-27 | 276-290 | 1669.0 | 1668.8 | 1668.9 |
| K32-33 | 349-370 | 2417.7 | 2417.5 | 2417.6 |
| | | | | |

- 5 GP=Glycopeptide
 ND=Not detected (m/z 2+ below scan range)
 Human ATIII
 ID=Incomplete digestion product
- phATIII=Human Plasma ATIII
 tgATIII=Transgenic (goat)

The glycopeptide peak retention times (glycopeptide peaks are labeled with K#'s on Figure 4) did not vary significantly from those of the plasma derived protein, but the chromatograms show that peak widening and peak splitting did occur due to the variability in the oligosaccharide residues attached to the glycopeptides.

Based on the LC/MS, peptide mapping, and carbohydrate composition data, the only differences observed between the tgATIII and the plasma derived hATIII arise due to the different patterns of glycosylation. Despite the presence of N-Acetylgalactosamine (GalNAc) in the monosaccharide analysis, no O-linked glycosylation was present on the transgenic protein, suggesting the presence of oligosaccharides different than found on the plasma derived human ATIII.

The major glycoform at each glycosylation site of the plasma derived (pATIII) is a complex type oligosaccharide and has a mass corresponding to HexNAc4, Hex5, NANA2 which is in agreement with the reported structure GlcNAc4, Man3, Gal2, NANA2. With the exception of Asn₁₅₅, located on K15, the major glycoforms on tgATIII contain oligosaccharides of the complex type. The major glycoform at each of the complex oligosaccharide containing sites on tgATIII has a mass corresponding to HexNAc4, Hex6, NANA1 which is in agreement with the structure GlcNAc4, Fuc1, Man3, Gal2, NANA1 with the second most abundant form being the disialylated form of the same structure, HexNAc4, Hex6, NANA2 which is in agreement with the structure GlcNAc4, Fuc1, Man3, Gal2, NANA2.

TgATIII contains a significant amount of oligomannose type and hybrid forms at Asn₁₅₅ and only a very low level of hybrid structures at the other locations. Oligomannose type structures are more primitive structures that are remodeled into the complex type oligosaccharides in the endoplasmic reticulum. Oligomannose structures display masses ranging from Hex5 to HexNAc2, Hex9. These values agree with structures comprised of 5 up to GlcNAc2, Man9, with only the number of mannose residues

varying. Hybrid oligosaccharides contain elements of complex oligosaccharides on one antenna of an individual glycosylation site and components of oligomannose type oligosaccharides on the other antenna. The N-linked glycosylation for tgATIII was much
5 more heterogeneous than phATIII, with a higher degree of fucosylation and more varied sialylation (Table 3). Several glycoforms with a mass difference of 41 were observed by LC/MS which can be accounted for by the substitution of a Hexose residue by a HexNAc (Tables 4). In view of the monosaccharide
10 composition (Table 1) and the lack of O-linked glycosylation (based on comparison of the observed vs theoretical mass for all peptides other than those containing an N-linked site) this could be accounted for by the substitution of one or more galactose residues by GalNAc. This result was confirmed by fragmentation
15 analysis mass spectroscopy of isolated glycopeptides. Figure 5 is an example of the mass spectrometry data for one of the individual glycopeptide residues from phATIII. It's pattern is fairly simple. In contrast, Figure 6 is an example of the same glycopeptide from tgATIII. The increased complexity of the
20 pattern is obvious, and can be deciphered into specific structures as described above.

Several glycoforms with mass differences of 16 mass units were also identified. The difference is explained by the presence of an oxidized form of sialic acid, N-Glycolylneuraminic acid
25 (NGNA) in place of N-Acetylneuraminic acid (NANA). NGNA is a common form of sialic acid found in goats. Approximately 25% of the sialic acids found in tgATIII are NGNA. Approximately 25% of goat plasma ATIII sialic acid is NGNA (Figure 7).

Thus, we have determined that; (1) one of the four
30 glycosylation sites on tgATIII has mainly high mannose (oligomannose) and hybrid type oligosaccharide structures, whereas the phATIII has biantennary, complex oligosaccharides on each of the four sites; (2) the complex oligosaccharides of tgATIII are not fully sialylated, whereas the phATIII
35 oligosaccharides are fully sialylated; (3) the tgATIII has a percentage of its sialic acid that is NGNA whereas the phATIII

has only NANA; and (4) tgATIII contains N-acetylgalactosamine on its N-linked oligosaccharides and the phATIII does not; and (5) the tgATIII has fucose on its proximal GlcNAc on each of the three sites having complex oligosaccharides, whereas the phATIII has only a very small amount of fucose on any site.

The tgATIII exhibits a faster clearance time in rabbits, mice and monkeys than does phATIII. Twenty ug samples of test ATIII was injected via the tail vein and residual ATIII determined using an ELISA assay which has little cross reactivity with mouse ATIII. The pattern shown in Figure 8 for clearance in mice mimics the pattern found for the same materials in rabbits. The clearance appears to be bimodal and is approximately 10 times faster than for hATIII. *In vivo* clearance was also examined in a monkey model system. Both trace and high levels of radioiodinated ATIII were injected and detected in plasma samples by counting in a gamma counter. The clearance pattern of tgATIII in monkeys indicated only a 4 to 5-fold faster clearance (Figure 9) from the circulation than the phATIII and could also be defined by a biphasic mechanism.

Early experiments indicate that the tgATIII may have a stronger affinity for heparin than the phATIII. This would be important since ATIII inhibits thrombin at inflammation or injury sites by binding to heparan sulfate in the endothelial layer of the vasculature. Once bound its affinity for thrombin is enhanced 1000-fold and it binds to and irreversibly inhibits thrombin.

THE INVENTION CLAIMED IS:

1. Transgenically produced antithrombin III comprising a monosaccharide composition which includes GalNAc.
2. Transgenically produced antithrombin III comprising a monosaccharide composition which includes GalNAc and which lacks O-linked glycosylation.
3. Transgenically produced antithrombin III comprising a monosaccharide composition of Fuc, GalNAc, GlcNAc, Gal, Man, and NANA/NGNA.
4. Transgenically produced antithrombin III having one of its glycosylation sites comprising oligomannose and/or hybrid oligosaccharide structures.
5. Transgenically produced antithrombin III comprising a monosaccharide composition which is primarily an oligomannose or hybrid type structure on one site and complex oligosaccharide on the remaining 3 sites.
6. Transgenically produced antithrombin III comprising a monosaccharide composition which is partially sialylated.
7. Transgenically produced antithrombin III comprising a monosaccharide composition including sialic acid which includes NGNA.
8. Transgenically produced antithrombin III comprising a monosaccharide composition which includes a fucose on its proximal GlcNAc on each of the sites having complex oligosaccharides.

9. The transgenically produced antithrombin III of claims 1, 2, 3, 4, 5, 6, 7 or 8 wherein the antithrombin III is transgenically produced in a mammal.
- 5 10. The transgenically produced antithrombin III of claim 1, 2, 3, 4, 5, 6, 7 or 8 wherein the antithrombin III is transgenically produced in a goat.
- 10 11. A transgenic method for producing antithrombin III in mammalian milk, comprising:
- a. producing a transgenic mammal that expresses a transgene which encodes a human antithrombin III with a monosaccharide composition which includes GalNAc;
 - b. collecting milk from the transgenic animal which
15 contains the human antithrombin III; and
 - c. isolating the human antithrombin III from milk.

1/11

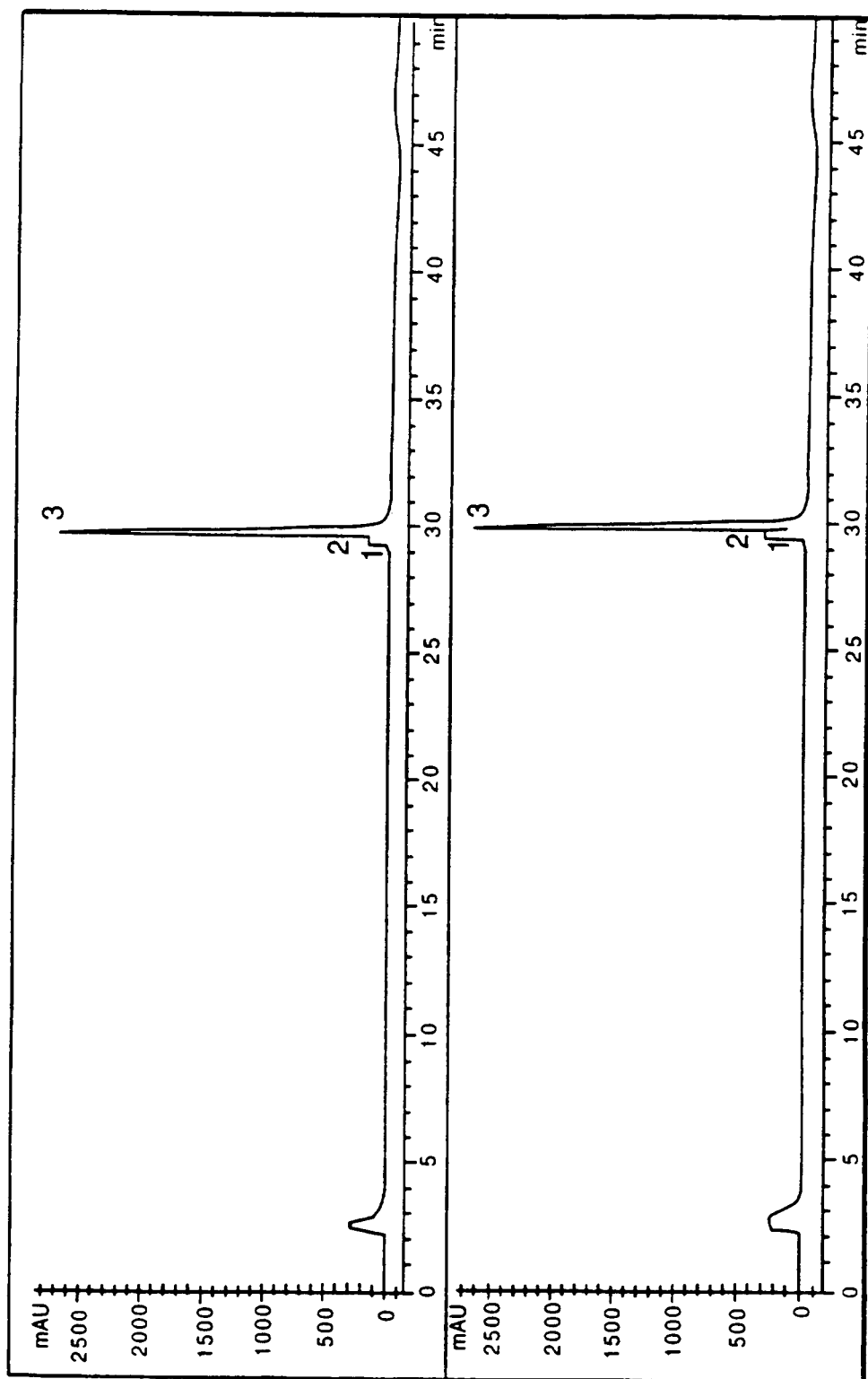


Figure 1

SUBSTITUTE SHEET (RULE 26)

2/11

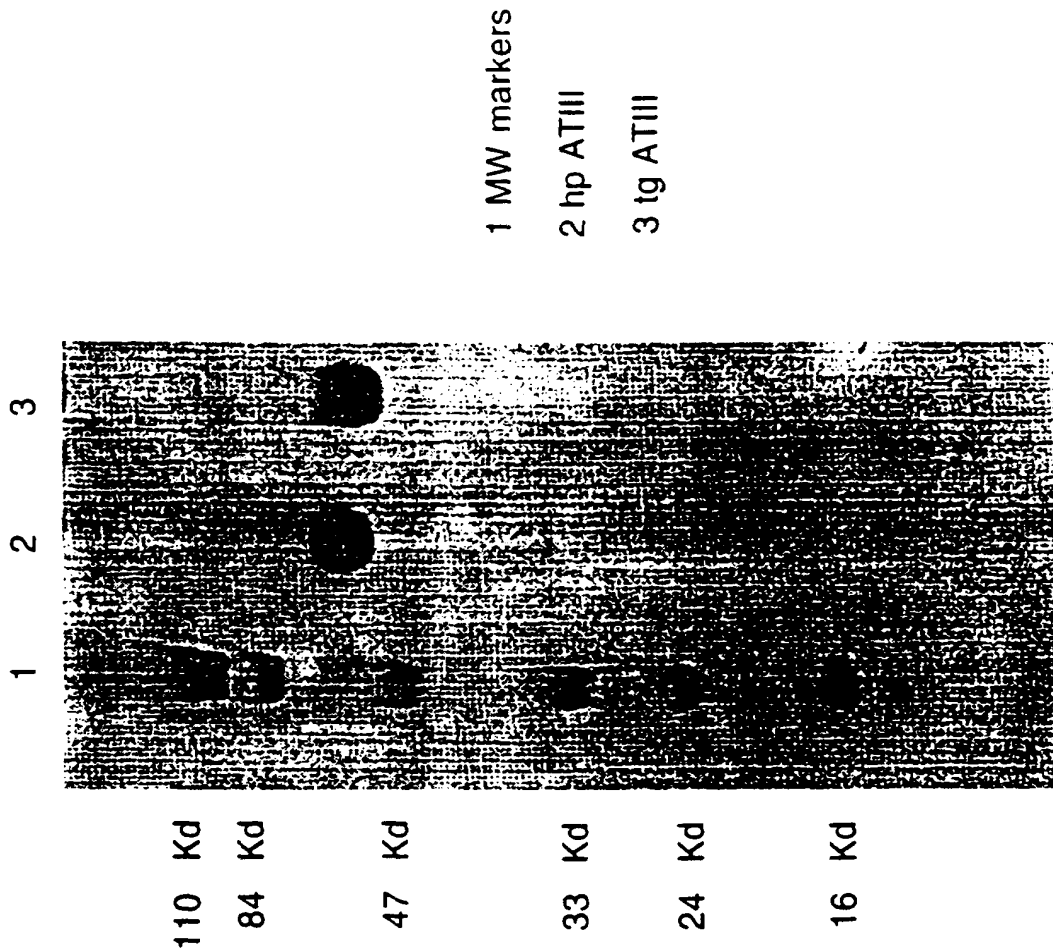


Figure 2

SUBSTITUTE SHEET (RULE 26)

3/11

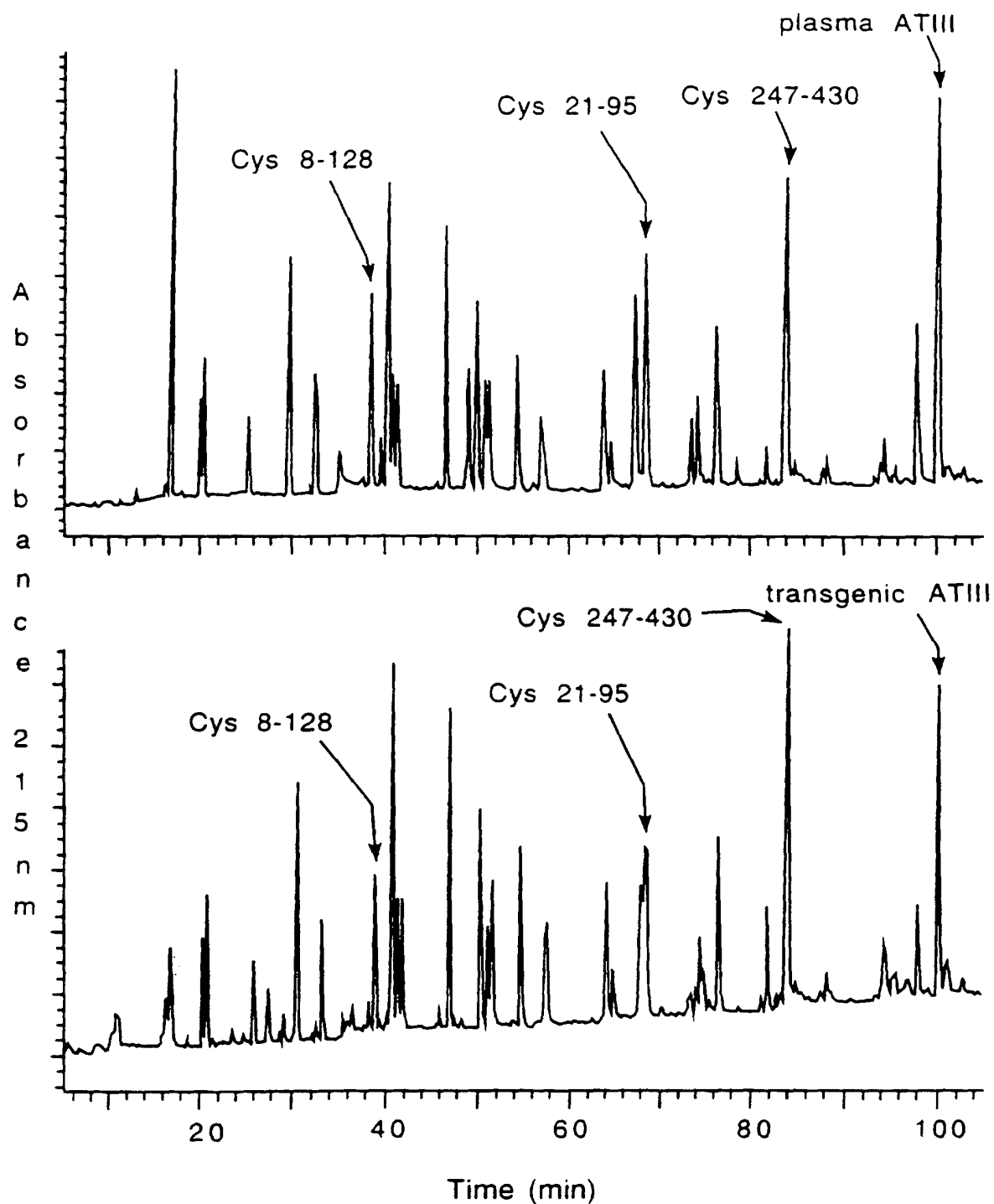


Figure 3

SUBSTITUTE SHEET (RULE 26)

4/11

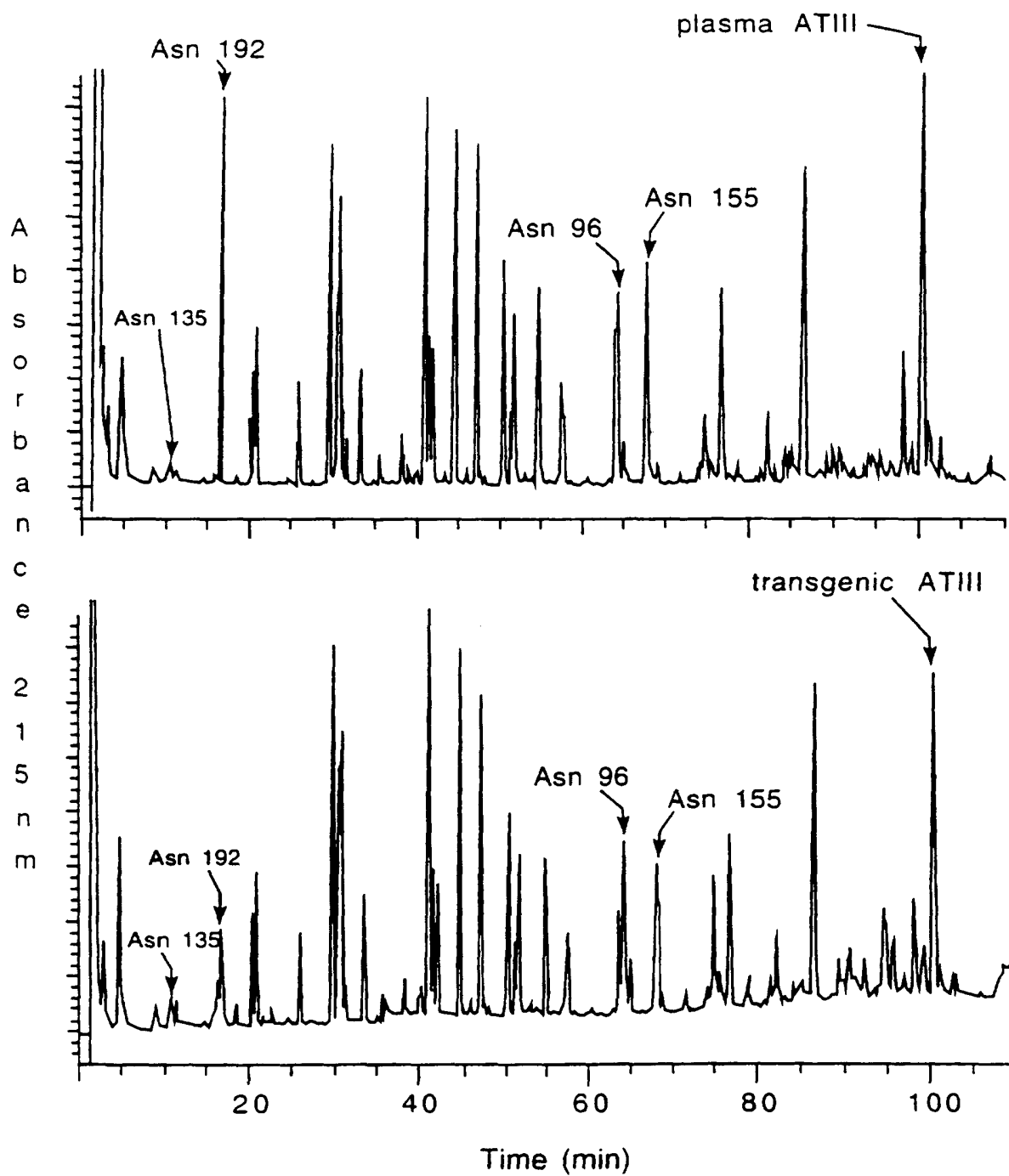


Figure 4

SUBSTITUTE SHEET (RULE 26)

5/11

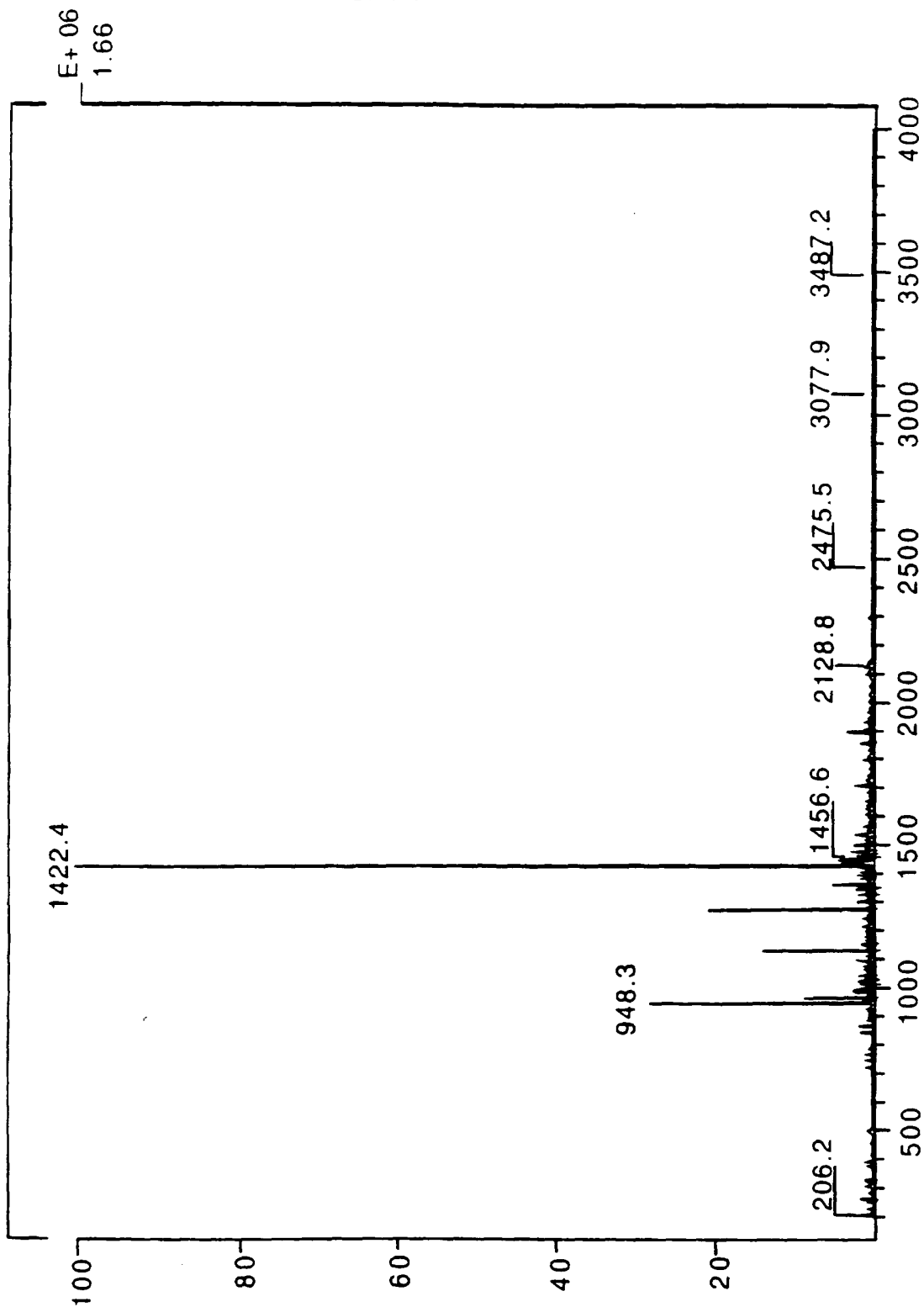


Figure 5

SUBSTITUTE SHEET (RULE 26)

6/11

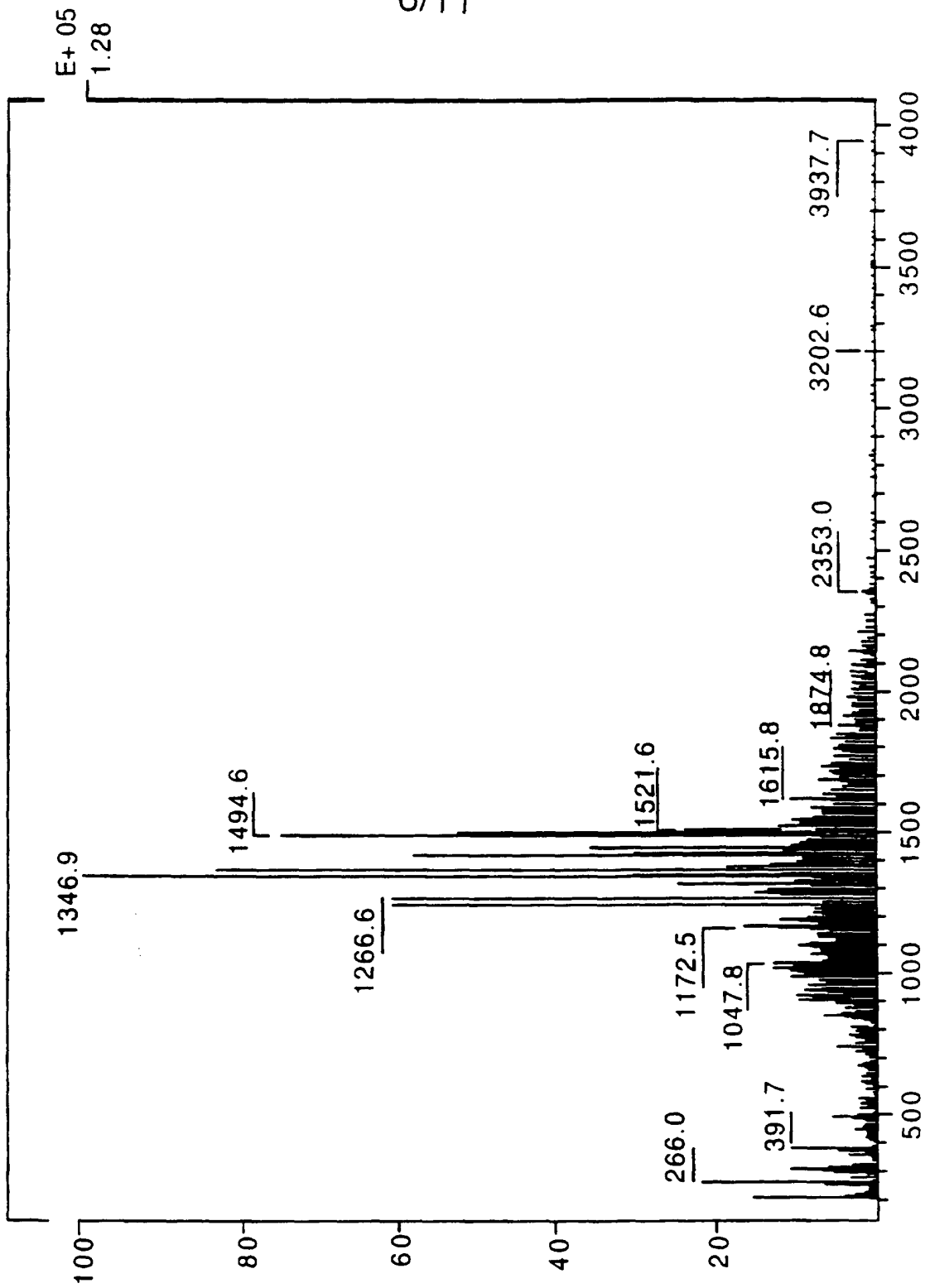


Figure 6

SUBSTITUTE SHEET (RULE 26)

7/11

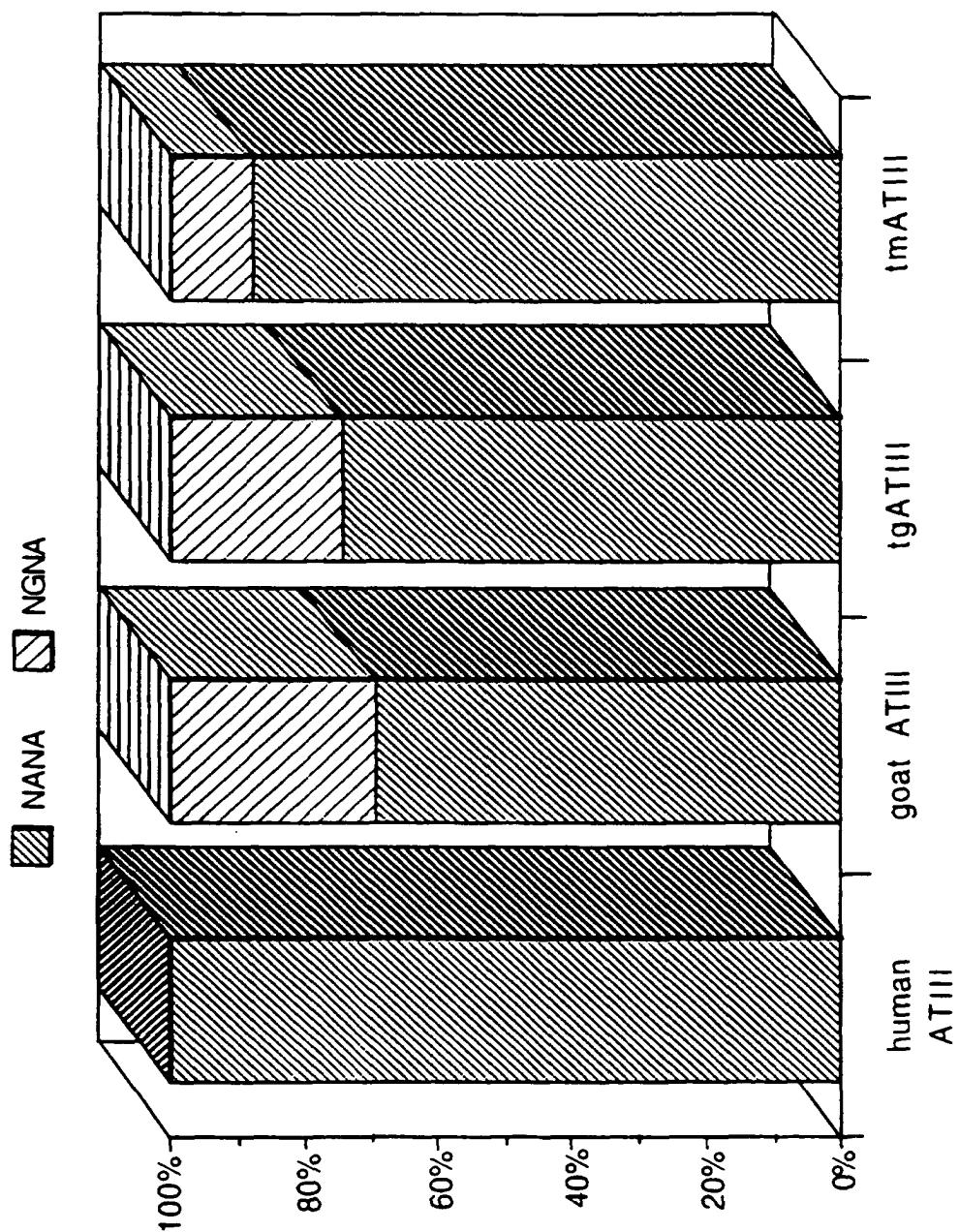


Figure 7

SUBSTITUTE SHEET (RULE 26)

8/11

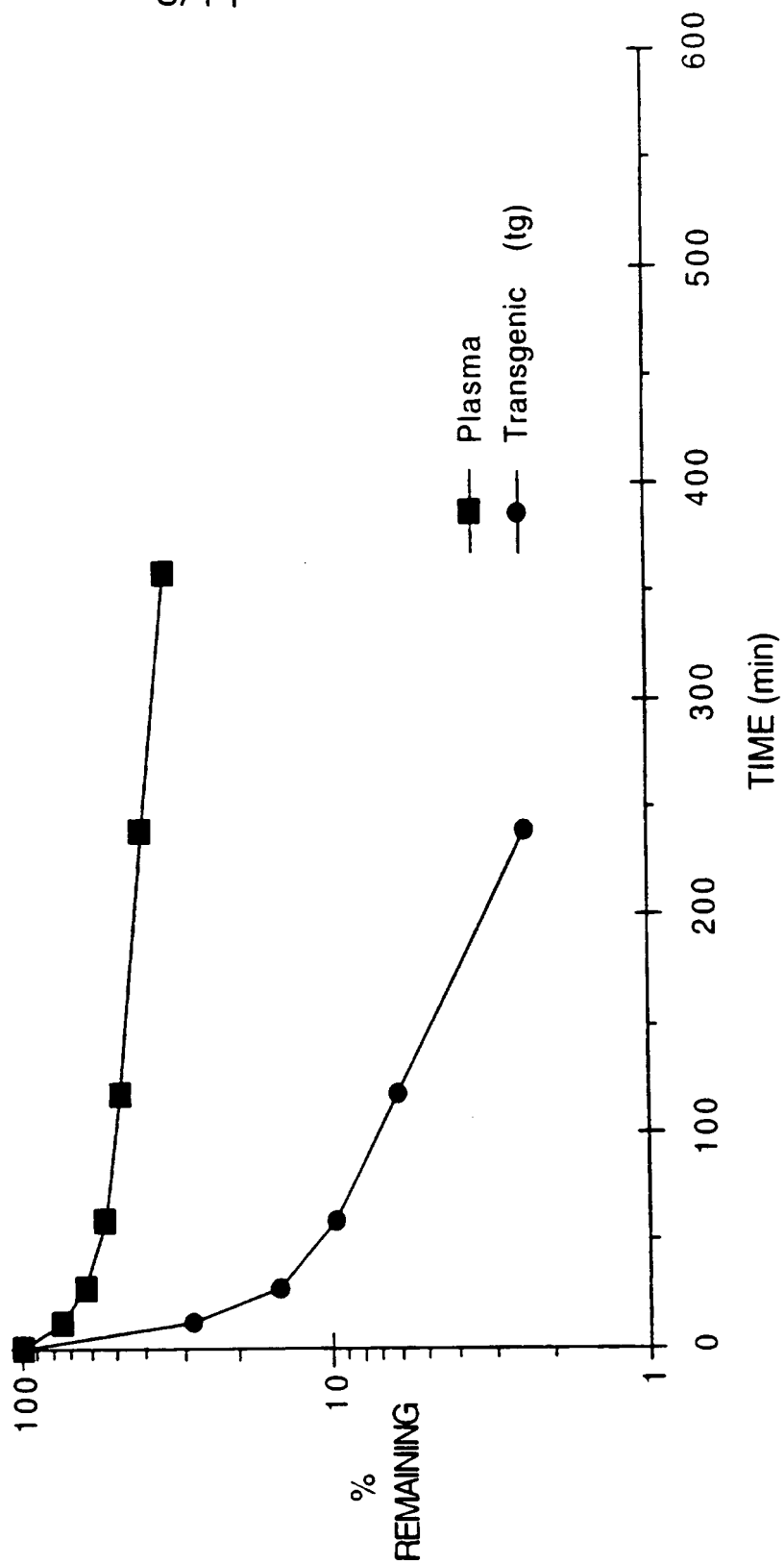


Figure 8

SUBSTITUTE SHEET (RULE 26)

9/11

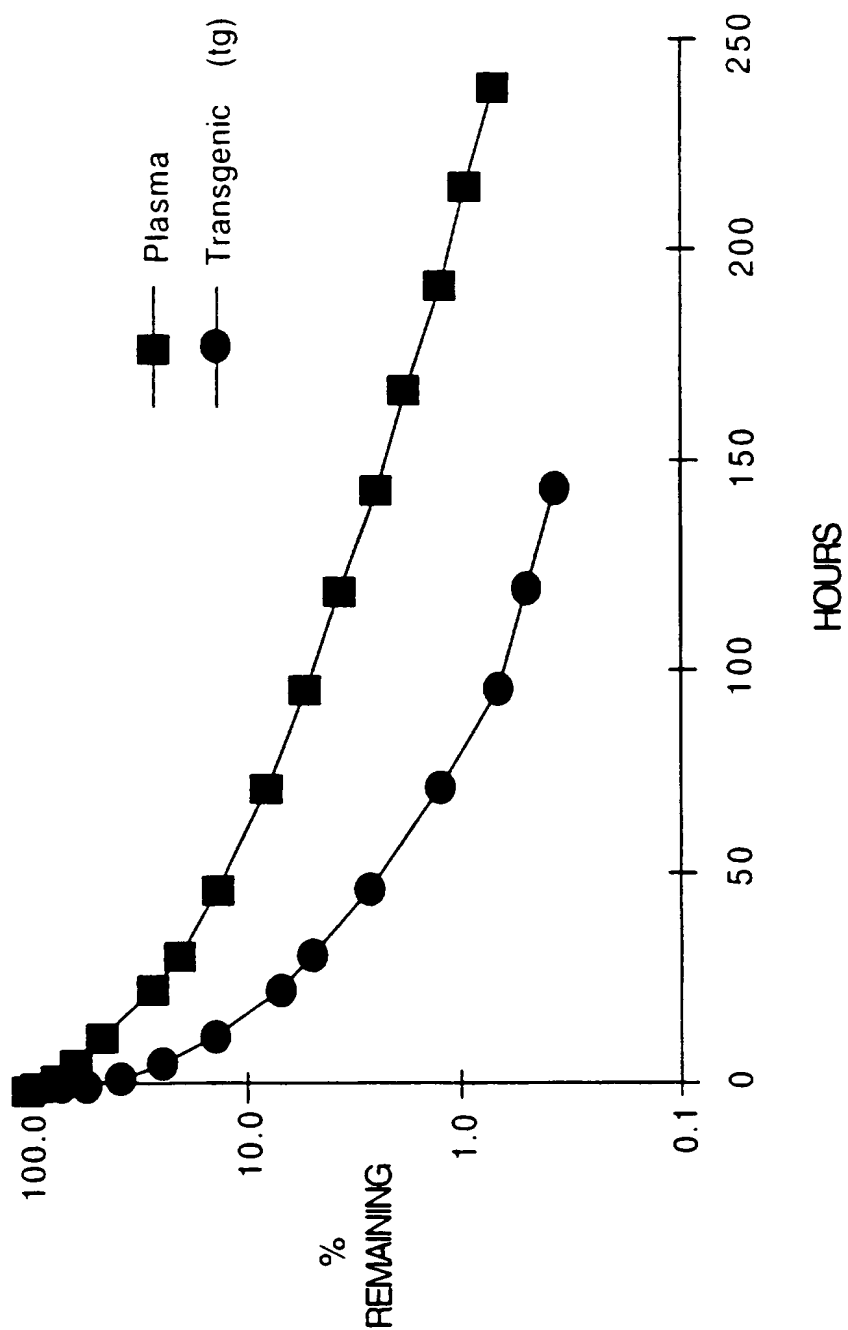


Figure 9

SUBSTITUTE SHEET (RULE 26)

10/11

Sequence Listing

agaatttaccccaagatctcaaagaccactgaataactaaagagacctcattgtggttac -263
 aataatttggggactggggccaaaacttccgtgcatcccagccaagatctgtagctactgg -203
 acaatttcatttcctttatcagattgtgagttattcctgttaaaatgctccccagaattt -143
 ctggggacagaaaaataggaagaattcatttcctaatacatgcagatttctaggaattcaa -83
 atccactgtttggttttattttcaaaccacaaaattagcatgccattaaatactatatataa -23
 acagccactaaatcagatcattATCCATTGAGCTTCTCCTTCACTTCTTCTCCTCTACTT 37
 TGGAAAAAAGgtaagaatctcagatataaatttcagtgatctgctactcatctttatttt 97
 ggactagggttaaaatgtagaaagaacataattgcttaaaatagatcttaaaaaataagggt 157
 gtttaagataagggtttacactattttcagcagatatgttaaaaaatagaagtactataa 217
 agacttgataaaaaattataggtgactgcaa.....tttttgccat 277
 gaggtttgcaggatcttgggtccctgaccagggatcaaacctgcactcccctggaagcat 337
 ggagtcttgacatttgtattatacactatcttgggttccttttaaagggaagtaatttt 397
 acttaazataagaaaatagattgacaagtaatacgtgtttcctcatcttcccattcacag 457
 GAATCGAGAGCCATGAAGGTCCTCATCCTTGCCTGTCTGGTGGCTCTGGCCATTGCAAGA 517
 **** M K V L I L A C L V A L A I A R 16
 GAGgtaataacagaaaaaatgttgaaataatagactagtactgtctgcctatgtgtagaa 577
 E 17
 atcacattaccaacatcataaatgtataaataatgcacaatctcagattttattttttaat 637
 gctaagaaagtcattttatgttcatccactatctcaacagtatcctataggaccacaactc 697
 tgggtcaagtgttttctatagtattgtaccatctgtaccatcaattcctaagaaaaagg 757
 aaaagaaaccaataagcaacagaccaacaagaaggaacacagacaagaacaaaaaatgag 817
 taatattgtacaaatacaattgcacg.....ctgcaggaactaaa 877
 gtgttttttttctctctcttttttagCAGGAAGAACTCAATGTAGTCGGTGAGgtaagat 937
 Q E E L N V V G E 26
 atttttatacaaagaaaaaaattaattttaactgtaaaatagtaacagactctgatgatct 997
 agcagaaaactcagctaattgtcaattttttatttttcttttatagACTGTGGAAGCCTT 1057
 T V E S L 31
 TCAAGCAGTGAGgtaagataatgttcattcagaggcaatttcccagatttagagcaataa 1117
 S S S E 35
 aacactgtattatctttttgtgttacattaattggcaaccactccagtactctt..... 1177
gacaaatatgaatttttcttttaaagctaaacctgattttattttt 1237
 atttttccaaagGAATCTATTACACACATCAATAAGgtaaaaccttcatatttaaactga 1297
 E S I T H I N K 43
 catttttaaaaatttcatgtttgattttttataaacagcatttctttatgtgtgatttttt 1357
 tttttaccagAAAATTGAGAAGTTTCAAAGTGAGGAACAACAGCAAACAGAGgtaatttg 1417
 K I E K F Q S E E Q Q Q T E 57
 ttcactatgagtatattttgagaagtattatgaaacataacacataaaaagattttataata 1477
 attatgttcagtctaagaatggtaataataagtgtcagtgtgaagaatgaaaactttgaca 1537

Figure 10 a

SUBSTITUTE SHEET (RULE 26)

11/11

| | |
|---|------|
| aaatgaaaaatttttaagatagaaacacattttttaaacacataaatcaaattttcagagta | 1597 |
| tagaataaaatacccaagaataaactactgggtatattcatttttactaatgggtatacctggct | 1657 |
| ttaataaatgcatattagtaggaacaattccagactagggtgatccccttatttcta | 1717 |
| atgatggatattgctgatgaaagacagtagggtgacagtgtggcactaatcctaatt..... | 1777 |
|aaatggaagatttttctttctctctcttactgaattatgttttaa | 1837 |
| aaagaggaggataattcatcatgaataacaattataactggattatggactgcaaaggca | 1897 |
| ttggttttcccttctttccagGATGAACTCCAGGATAAAATCCACCCCTTTGCCCAGGCAC | 1957 |
| D E L Q D K I H P F A Q A Q | 71 |
| AGTCTCTAGTCTATCCCTTCACTGGGCCCATCCCTAACAGCCTCCACAAAACATCCTGC | 2017 |
| S L V Y P F T G P I P N S L P Q N I L P | 91 |
| CTCTTACTCAAACCCCTGTGGTGGTGCCGCCTTTCTTTCAGCCTGAAATAATGGGAGTCC | 2077 |
| L T Q T P V V V P P F L Q P E I M G V P | 111 |
| CCAAAGTGAAGGAGACTATGGTTTCTAAGCACAAAGAAATGCCCTTCCCTAAATATCCAG | 2137 |
| K V K E T M V P K H K E M P F P K Y P V | 131 |
| TTGAGCCCTTTACTGAAAGCCAGAGCCTGACTCTCACTGATGTTGAAAAGCTGCACCTTC | 2197 |
| E P F T E S Q S L T L T D V E K L H L P | 151 |
| CTCTGCCTCTGGTCCAGTCTTGGATGCACCAGCCTCCCCAGCCTCTTTCTCCAACCGTCA | 2257 |
| L P L V Q S W M H Q P P Q P L S P T V M | 171 |
| TGTTTCTCTCCTCAGTCCGTGCTGTCCCTTTCTCAGCCCAAAGTTCTGCCTGTTCCCCAGA | 2317 |
| F P P Q S V L S L S Q P K V L P V P Q K | 191 |
| AAGCAGTGCCCCAGAGAGATATGCCCATCCAGGCCTTTCTGCTGTACCAGGAGCCTGTAC | 2377 |
| A V P Q R D M P I Q A F L L Y Q E P V L | 211 |
| TTGGTCTGTCCGGGGACCCTTCCCTATTCTTgtaagtctaaatttactaactgtgctgt | 2437 |
| G P V R G P F P I L | 221 |
| ttaacttctgatgtttgtatgatatttgagtaattaagagccctacaaaaaaatcaataa | 2497 |
| tgaatggttccaaaataagcatagctgagattaatgattctcagcattagttataaatag | 2557 |
| aataagctggaaaaccttcacctcccctccaccaccagatccc..... | 2617 |
| ...aaaaacaaaatactgaagatgcttatttcaatactcagggaaaattttcttgccaaa | 2677 |
| aaggcaagaattgtataattcattcacttattttatttttttaatttttaagGTCTAAG | 2737 |
| V * | 222 |
| AGGATTTCAAAGTGAATGCCCCCTCCTCACTTTTGgt.....caa | 2797 |
| attggaaatgggggtgagatgaagagttataacatataactaaatggacattgttctcta | 2857 |
| ttccacagAATTGACTGCGACTGGAAATATGGCAACTTTTCAATCCTTGCATCATGCTAC | 2917 |
| TAAGATAATTTTAAATGAGTATACATGGAACAAAAAATGAACTTTATTCTTTATTTA | 2977 |
| TTTTATGCTTTTTCATCTTAATTTGAATTTGAGTCATAAACCATATACTTTCAAATGTT | 3037 |
| AATTCACATTAGCATAAAAGTTCAATTTTAACTTGAAATATCATGAACATATCAAATT | 3097 |
| ATGTATAAAAAATAATTTCTGgaattgtgattattatttctttaagaatctatttccctaac | 3157 |
| cagtcatttcaataaattaacccttaggcataatttaagtttcttgctttatttatattt | 3217 |
| ttaaaaatgaaattgggtctctttattgttaacttaaatattctttgatgttaaaaatag | 3277 |
| ctgtgaaaaa | 3287 |

Figure 10 б

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02420

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 9/64

US CL : 530/350; 435/226; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/226; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, MEDLINE, EMBASE, BIOSIS, SCISEARCH search terms: human antithrombin III, transgenic, knockout, null, goats, mammary gland, milk, antithrombin III, mammal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | US 5,366,894 A (A. J. CLARK ET AL.) 22 November 1994, column 3, lines 63-66. | 9-11 |
| Y | US 4,873,316 A (H. MEADE ET AL.) 10 October 1989, column 2, lines 57-61. | 9-11 |
| Y | US 4,517,294 A (S. C. BOCK ET AL.) 14 May 1985, column 5, lines 59-65, and Figure 2. | 9-11 |
| Y | US 4,632,981 A (S. C. BOCK ET AL.) 30 December 1986, column 3, lines 64-68, and column 4, lines 1-3. | 1- 8 |

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

| | | |
|---|-----|--|
| * Special categories of cited documents: | *T | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* document defining the general state of the art which is not considered to be of particular relevance | *X* | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *E* earlier document published on or after the international filing date | *Y* | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *Z* | document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | | |
| *P* document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search

15 MAY 1996

Date of mailing of the international search report

23 MAY 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MARK SPENCER

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*